THE MECHANISM OF HETEROKARYOTIC GROWTH IN *VERTICILLIUM DAHLIAE*[1]

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ABSTRACT

Heterokaryons of *Verticillium dahliae*, forced between complementary auxotrophs, were stable at 21° and resembled the wild type morphologically. In such heterokaryons the hyphal cells were predominantly uninucleate, and no nuclear migration from cell to cell was observed. Heterokaryosis was apparently confined to binucleate, interhyphal, anastomosed cells that arose 1–2 mm behind the colony front. Such anastomosed cells thereby fed and maintained large homokaryotic areas including the colony edge. This stable mosaic colony is in sharp contrast to the heterokaryon of Neurospora.—Heterokaryons of *V. dahliae* cannot continue growth at 30° because the high temperature prevents hyphal anastomosis. Heterozygous diploids sector out from heterokaryons after 8–12 days at 30°. Interhyphal anastomosed cells are apparently the site of karyogamy.

THE term heterokaryosis was defined by HANSEN and SMITH (1932) as "the condition of a cell containing two or more genetically different nuclei. . . ." Although HANSEN and SMITH worked with Botrytis, most of our information on the heterokaryotic fungal colony is based on work with Neurospora (DODGE 1935; BEADLE and COONRADT 1944; PITTENGER and ATWOOD 1956). A Neurospora heterokaryon has three salient features: (1) the hyhal cells are multinucleate, (2) nuclei can migrate from cell to cell and the heterokaryotic cells themselves can grow and divide, and as a result (3) heterokaryosis extends to the apical cells of a colony. These features may apply to many fungi; e.g., *Aspergillus nidulans* (PONTECORVO 1953), *Penicillium cyclopium* (REES and JINKS 1952) and Rhizoctonia (WHITNEY and PARMETER 1963). Dikaryons of *Schizophyllum commune* (SNIDER and RAPER 1958) and *Coprinus Lagopus* (SWIEZYNSKI and DAY 1960) represent a special form of this type of heterokaryon. Both PARMETER, SNYDER and REICHLE (1963) and DAVIS (1966), in their reviews of heterokaryosis, acknowledge that the Neprospora heterokaryon is probably not the only type of heterokaryon in fungi. However, they cite the lack of data that would suggest another form.

[1] This work was performed in cooperation with the Plant Sciences Department, Texas Agricultural Experiment Station, Texas A&M University.

In this paper we describe heterokaryosis in *Verticillium dahliae* Kleb. It does not fit the Neurospora type. We propose a second type of heterokaryosis to explain our data. The possibility of a similar type in other fungi is discussed. In addition, the origin and development of heterozygous diploid strains from heterokaryons are described.

MATERIALS AND METHODS

Strains: All strains of *V. dahliae* were derived from wild-type T9, which belongs to the T1 line described by SCHNATHORST (1966). T9 was originally isolated from cotton, in which it causes severe wilt and defoliation. It is typical of *Verticillium dahliae* because it produces heavy-walled, black resting structures called microsclerotia.

Stock cultures were kept at 21° and transferred every 2 weeks as multiple hyphal tip sections to maintain uniformity. Cultures were also stored at 5° on silica gel using the method of PERKINS (1962).

Media: Minimal agar medium contains the following: KH_2PO_4, 1.4 g; K_2PO_4, 1.7 g; Na_2SO_4, 3.2 g; $Ca(NO_3)_2.4\ H_2O$, 0.4 g; KNO_3, 4.6 g; $MgSO_4.7H_2O$, 0.7 g; NaCl, 0.5 g; minor element solution, 1.0 ml; sucrose, 20 g; agar, 20 g; distilled water to one liter. The minor element solution contained (in g/1): $ZnSO_4.7H_2O$, 1.0; $MnCl_2.4H_20$, 1.0; H_3BO_3, 1.0; $FeCl_3.6H_2O$, 0.5; $CuSO_4.5H_2O$, 0.1; KI,0.1; $Na_2\ MoO_4.2H_2O$, 0.1. Supplementations of this minimal medium were made at the following concentrations: amino acids, 100 mg/1; nitrogenous bases, 10 mg/1; vitamins 10 mg/1.

Auxotrophic mutant stocks were maintained on appropriately supplemented minimal agar or on potato carrot dextrose agar (BIRD 1966; PUHALLA 1973).

Mutant induction: An aqueous suspension of T9 condia at a concentration of 10^6 spores/ml was irradiated with short wave ultraviolet light from a model R51 Mineralight (Ultraviolet Products, San Gabriel, California).[1] The dosage (ca. 1800 ergs/cm^2) was adjusted to kill 95–99% of the spores, which were then spread on plates of potato carrot dextrose agar. Colonies from surviving conidia were replica plated with sterile velvet to minimal medium. Auxotrophs were isolated, cloned and tested for their specific requirements. Some auxotrophs were irradiated again to produce strains with two or three requirements.

The symbols used in this paper for these auxotrophic mutants and their requirements are: *nic*, nicotinamide; *hist*, histidine; *iv*, isoleucine and valine; *ser*, serine; *arg*, arginine; *met*, methionine; *ad*, adenine; *leu*, leucine; *adhi*, adenine and histidine.

Heterokaryon synthesis: Heterokaryons were forced between two strains, each carrying two or three complementary, auxotrophic mutations. Small blocks, 2 mm on a side, were cut from actively growing cultures of each strain and placed in contact on minimal agar. The pairings were incubated at 21°. Growth from the pairings was assumed to be heterokaryotic. These heterokaryons were stable and have been maintained on minimal medium at 21° by mass transfer of hyphae for several months. For most experiments, however, the heterokaryons were freshly synthesized from the component strains.

Pairings were also made in which cellophane (#124-PD Cellophane, DuPont de Nemours & Co., Wilmington, Del.) was interposed between the two complementary auxotrophs. We found that the fungus did not penetrate this cellophane during the course of these studies. However, the cellophane did not prevent the passage of small molecules such as glucose, vitamins, amino acids and nitrogenous bases.

Heterokaryon analysis: Conidia from a heterokaryon were spread on minimal agar medium or on minimal agar medium supplemented with the requirements of either component. Because the conidia are uninucleate, their nuclear type was thereby directly determined. If there is no nuclear selection during conidial formation, the proportion of nuclei in the conidia should reflect the proportion in the hyphae.

[1] Mention of a trade name, a proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

The distribution of the two auxotrophic components in the heterokaryotic colony was determined from mycelial blocks that were taken in sequence along radii. These blocks were 1500 μ wide and 500–2000 μ long as measured along the radius of the colony. A 1500 μ wide block from the edge of the colony carried 5–10 hyphal tips. Three radii from each colony were sampled. All the blocks from the first radius were transferred to minimal, all those from the second radius to minimal supplemented with the requirements of one auxotrophic component, and all those of the third to minimal supplemented with the other component's requirements. At least three replicate colonies of each heterokaryon were sampled. The entire edge (500 μ) of three heterokaryotic colonies 3 cm in diameter was also sampled and transferred to the three media listed above. All the edge blocks from one colony were transferred to the same medium.

Diploid formation: Heterokaryons that had grown to a diameter of 3 cm at 21° were transferred to 30°. At this temperature, growth of the heterokaryons ceased. After 8–12 days small outgrowths appeared at the periphery of the colonies. This growth was diploid and heterozygous for the auxotrophic mutations of the heterokaryons.

Cytology: Nuclear counts and size determinations of conidia were made as described previously (PUHALLA 1973). At least 50 conidia were sampled in every determination.

Colonies of T9 were grown on 2% water agar medium and on minimal medium at 21° and 30°. On both media the diameter of colonies increases at the same rate, but the growth is much thinner on the water agar medium. Colonies up to 3 cm diameter were scanned with a light microscope. The area where hyphal anastomoses began was easily seen on both media. However, a quantitative estimation of anastomoses was much easier and much more accurate on the water agar medium because of the fungus' thin growth. The number of anastomoses in at least 25 microscope fields were counted at each temperature. A culture of heterokaryon A (See Table 1) grown at 21° was also examined.

The hyphae of heterokaryons were also examined with a phase-contrast microscope. The heterokaryons were grown on cellophane over 2% water agar and incubated at 24°. Small squares of cellophane carrying mycelium were cut from various areas of the colony and inverted in a drop of distilled water on a microscope slide. The cellophane was then floated away from the mycelial mat, a cover glass was added and excess water was blotted away. This provided a very thin preparation in which the nuclei of the hyphae were clearly visible.

RESULTS

Auxotrophs: The percentage of auxotrophs among the survivors of ultraviolet irradiation was high, often 0.5–1.0. The range of auxotrophs found was unusual only in one respect: a high proportion (16%) required nicotinamide. The auxotrophs were stable and presumably were due to single nuclear gene mutations.

Heterokaryons at 21°: After a delay of several days, most pairings of complementary auxotrophs produced colonies that, except for a slower growth rate, resembled wild-type T9 (Figure 1). When cellophane was interposed between the paired blocks, there was no growth. Growth from the pairing was therefore considered to be heterokaryotic and not the result of cross-feeding.

The four heterokaryons studied most extensively are listed in Table 1. The nuclear proportions in the conidia of the heterokaryons are also shown. In most cases the nuclei of one component predominated. Although over 10^8 conidia from several heterokaryons were plated, no prototrophic conidia were found.

Individual hyphal tips that were removed from the heterokaryons at 21° were invariably auxotrophic and of one genotype. Mycelial sections, sampled from the edge 500–1000 μ of these heterokaryons, were also auxotrophic. Only sections taken from more than 1000 μ behind the colony edge were prototrophic; i.e., they grew on minimal medium. These findings are summarized in Table 2.

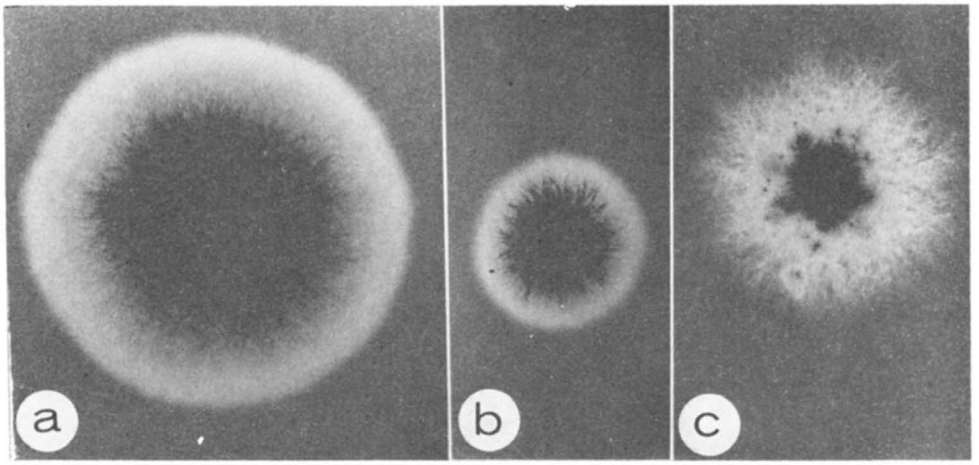

FIGURE 1.—Morphology of colonies of *Verticillium dahliae* grown at 21° for 11 days. (a) Wild-type T9. (b) Heterokaryon A. (c) Diploid derived from heterokaryon A.

TABLE 1

Characteristics of heterokaryons of Verticillium dahliae

Heterokaryon	Auxotrophic components X + Y*	Nuclear ratios in conidia, X:Y (no. conidia counted)	Growth rate in mm/day†
A	*nic-1 hist-3* + *iv-1 ser-1 arg-13*	5.8:1 (105)	1.7
B	*nic-1 hist-3* + *met-1 arg-4*	>420:1 (420)	1.1
C	*nic1 ad-6 leu-3* + *met-1 arg-4*	>610:1 (610)	1.1
D	*nic-1 hist-3* + *nic-1 met-3 arg-13*	3.9:1 (705)	2.4

* Numbers accompany symbols refer to isolate number, not to a specific gene locus.
† Growth rate measured from colonies increasing from 2cm to 3cm in diameter on minimal agar (minimal + nicotinamide for D) at 21°. T9 under these conditions grows at a rate of 3.8 mm/day.

TABLE 2

Distribution of components in heterokaryotic colonies (3 cm in diameter) of Verticillium dahliae *on minimal medium at 21°*

Heterokaryon*	Distance (μ) from colony edge to X	Y	Prototrophy	Section along colony edge; percentage of sections from which were recovered X	Y	Prototrophy
A	0	0	833	97	80	0
B	0	1000	1000	100	2	0
C	0	1000†	1000†	100	2	0
D§	167	333	1000	75	75	15‡

* See Table 1 for description of components X and Y of each heterokaryon.
† This measurement was from the dense, uniform colony edge, but there were numerous long hyphae extending up to 1 cm beyond this edge. Such hyphae were invariably component X.
‡ The prototrophic mycelial sections grew on minimal + nicotinamide only after a delay.
§ Heterokaryon D was grown on minimal medium + nicotinamide because X and Y shared the same nic mutation.

Table 2 also lists the results of sampling the edge (500 μ) of the entire heterokaryotic colony. Nearly the entire edge of heterokaryons B and C consisted of a single auxotrophic component, and no samples were prototrophic. Most of the sections from the edge of heterokaryons A and D carried both auxotrophic components, yet most of them did not grow on minimal medium. Those that did grow showed a delay of 4–6 days before growth began. Such a delay was comparable to that found in the original pairings of the complementary auxotrophs. By contrast, the prototrophic samples taken at least 1000 μ back from the edge, where hyphal anastomoses were frequent, grew on minimal medium without delay.

Heterokaryons were affected by the growth medium. When a heterokaryon grew on minimal medium agar supplemented with the requirements of the major auxotrophic component, the major component grew out from the heterokaryon and occupied the entire growing edge of the colony. Only the very center remained heterokaryotic. The behavior of heterokaryons on minimal medium supplemented with the requirements of the minor component was more variable. In heterokaryons with a very disparate nuclear ratio (heterokaryons A, B, and C), growth rate of the colony increased significantly. However, the distribution of the two auxotrophic components in the colony and at the edge was similar to that observed on minimal medium. In heterokaryon D with a more even nuclear ratio, the minor component occasionally outgrew the heterokaryon on supplemented minimal medium.

Colonies of T9 and heterokaryon A on minimal medium and on 2% water agar medium at 21° formed numerous anastomoses between hyphae. Typical anastomoses are represented diagrammatically in Figure 2 and were found only more than 800 μ behind the colony edge. This area corresponds to where prototrophic growth began in the heterokaryotic colony.

The nuclei in the H-shaped anastomosed cells could be seen with a phase-contrast microscope(Figure 3). Many of the anastomosed cells had two nuclei, one from each hypha involved in the anastomosis. Migration of one nucleus across the bridge to the other hypha was often observed.

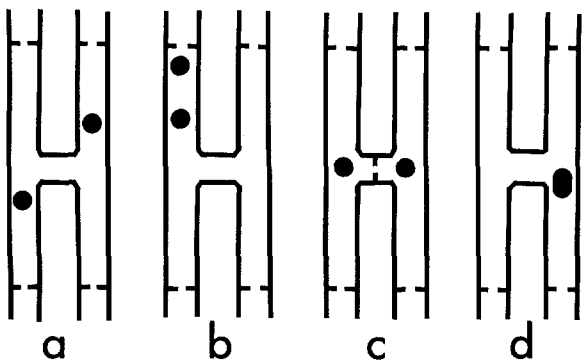

FIGURE 2.—Diagrammatic representation of anastomoses between hyphae of *Verticillium dahliae*. Nuclei are shown as closed circles. (a) no nuclear migration. (b) nuclear migration across the anastomosis bridge. (c) an anastomosis bridge with a septum. (d) a uninucleate anastomosed cell.

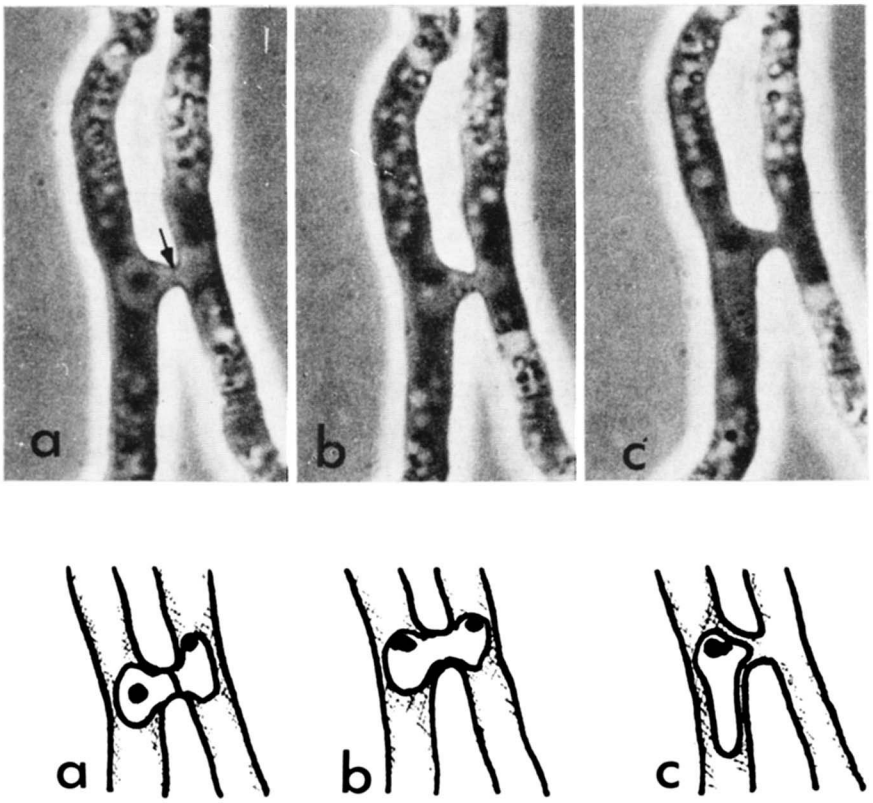

FIGURE 3.—Photo and interpretive drawing of karyogamy in an anastomosed cell formed between hyphae of *Verticillium dahliae*. (a) 0 min. The two nuclei are separated by a membrane (arrow). (b) 50 min. The two nuclei appear continuous. (c) 132 min. There is only one large nucleus which is situated to one side of the anastomosis bridge.

A likely instance of nuclear fusion in an anastomed cell is shown in Figure 3. In spite of repeated attempts, this was the only actual nuclear fusion observed. This may be explained in part by the short period of time in which fusion occurs. However, anastomosed cells containing only one nucleus (Figure 2d) were seen several times. These cells may be the consequence of such nuclear fusions. Migration of nuclei from the anastomosed cell into adjacent cells was not seen, even though the septa had a central pore (unpublished data). Growth or division of anastomosed cells was not seen at 21°. The cells of the heterokaryon that were not involved in anastomosis remained uninucleate.

Heterokaryons at 30 : When heterokaryons on minimal medium were transferred from 21° to 30°, they soon ceased growing. In contrast, T9 on minimal medium or the individual components on supplemented minimal medium continued to grow at 30° (Figure 4). Samples of mycelium taken from these heterokaryons after 8 days at 30° showed some changes in the distribution of the components, but samples from the edge remained auxotrophic (Table 3).

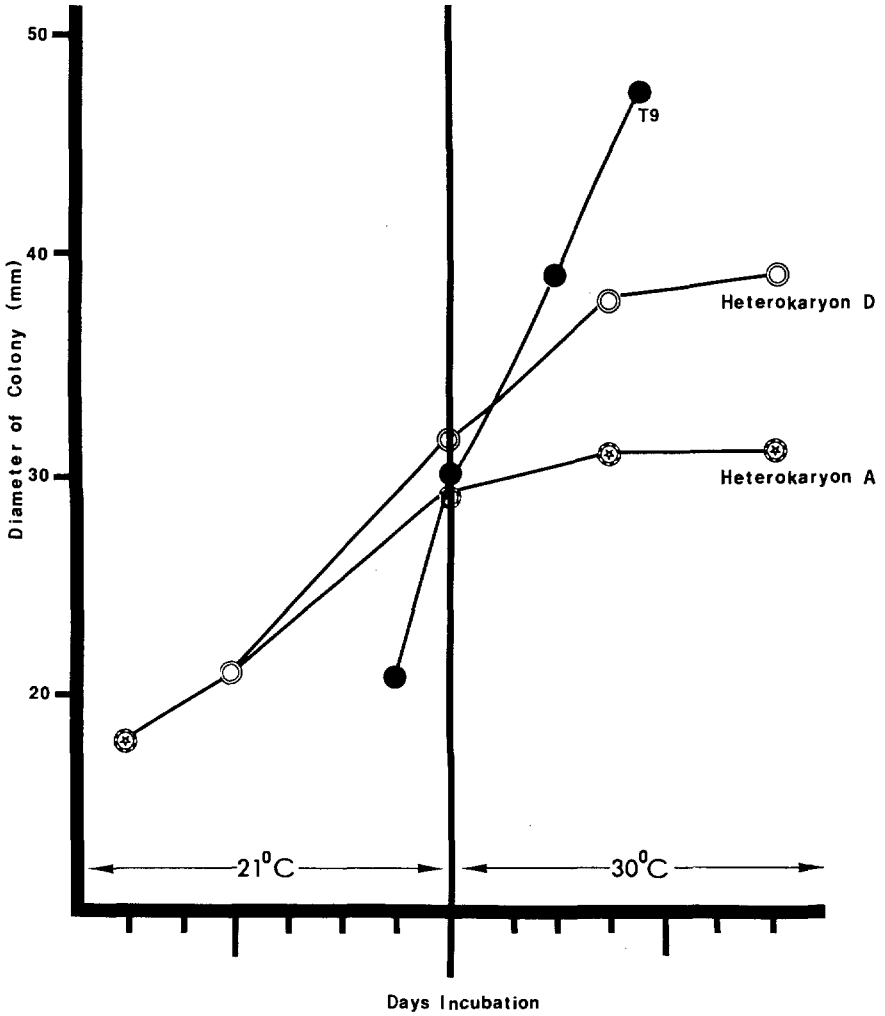

FIGURE 4.—Growth of heterokaryons A and D and wild-type T9 of *Verticillium dahliae* on minimal medium. Colonies were incubated at 21° until they attained a diameter of ca. 30 mm. They were then transferred to 30°. Each point is the average of 4 replications. Each division on the abscissa represents one day.

On water agar T9 colonies at 30° showed fewer anastomoses than at 21°. In the area of the colony 1–2 mm back from the front only 2.3 anastomoses per microscope field ($1.3 \times 10^5\ \mu^2$) were found at 30°, compared with 11.0 anastomoses per field at 21°. The density of growth at each temperature was comparable. The high temperature did not prevent hyphal branching, but it did reduce subsequent hyphal fusions. However, at either temperature anastomoses began around 800 μ behind the colony edge. Quantitative estimations of hyphal anastomoses on minimal medium were more difficult because of the heavier growth. In one trial only 4.3 anastomoses per field were found at 21° and no anastomoses whatsoever were found at 30°.

TABLE 3

Distribution of components in heterokaryons of Verticillium dahliae *on minimal medium after 8 days at 30°*

Heterokaryon*	Distance (μ) from colony edge to X	Y	Prototrophy
A	0	0†	0†
B	0	677	1000
C	0	2000‡	2667‡
D§	0	333	1000

* See Table 1 for description of components X and Y of each heterokaryon.
† Putative prototrophic diploid had already overgrown heterokaryon.
‡ This measurement was from the dense, uniform colony edge, but there were numerous long hyphae extending up to 1 cm beyond this edge. Such hyphae were invariably component X.
§ Heterokaryon D was grown on minimal medium + nicotinamide because X and Y shared the same nic mutation.

In heterokaryon A at 21° anastomoses were even more frequent than in T9 at 21°. Approximately 18.7 fusions per field (average of 9 fields) were found. As in the T9 colony, no anastomoses were found in the outer 800 μ of growth. Because heterokaryon A does not grow at 30°, a decrease in the number of anastomoses within it could not be assayed.

Diploids: After heterokaryons had been at 30° for 8–12 days, small outgrowths appeared on the periphery of the colonies (Figure 5). This growth was distinct from that of wild-type T9: the colony was brownish, the edge was irregular, the

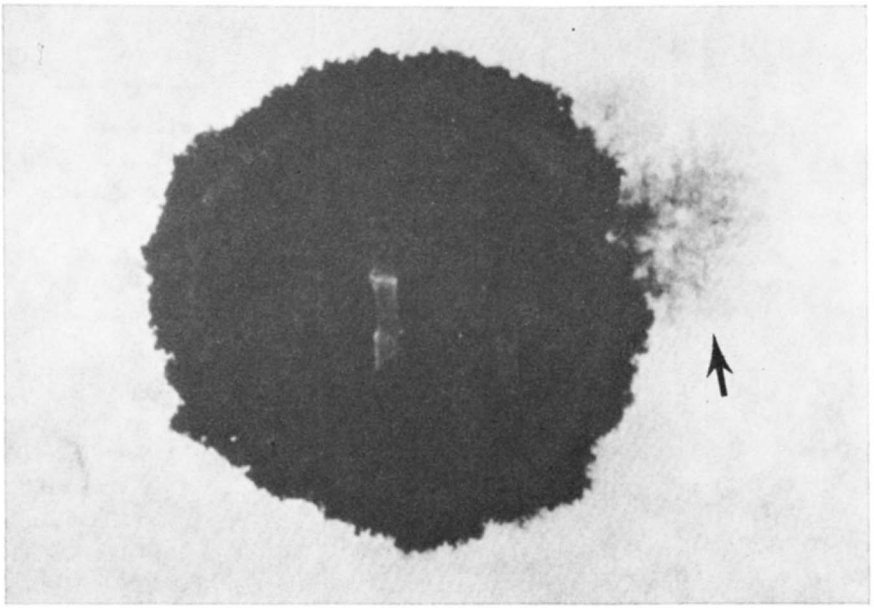

FIGURE 5.—Diploid growth at the periphery of a heterokaryotic colony of *Verticillium dahliae*. The heterokaryon was grown at 21° to a diameter of 30 mm and then transferred to 30° for 12 days.

TABLE 4

Strains recovered from the breakdown of putative heterozygous diploids of Verticillium dahliae

Genotype of putative diploid*	No. of sectors tested	Auxotrophic markers recovered	No. of auxotroph combinations Parental	Non-parental
nic-1 hist-3 / *met-1 arg-4*	32	*nic, arg, hist*	1	5
nic-1 hist-3 / *nic-1 met-3 arg-13*	14	*nic, met, hist*	1	4
nic-1 leu-3 / *met-1 adhi-1*	55	*nic, leu, met, adhi*	2	12
nic-1 hist-3 / *iv-1 ad-17*	30	*nic, hist, iv, ad*	0	10

* Genotype of the diploid is assumed from the auxotrophic mutations heterokaryon from which the diploid arose. Numbers accompanying symbols refer to isolate numbers, not to a specific gene locus.

hyphae were sinous and contorted, and microsclerotia were distributed irregularly (Figure 1).

The peculiar outgrowth from heterokaryons at 30° was probably diploid and heterozygous for the auxotrophic mutations in the components of the heterokaryon. Conidia of these putative diploids were uninucleate like the conidia of T9, but they were almost twice as long. The average size of T9 conidia was 6.4 $\mu \times 4.4\ \mu$; the average size of the conidia from five different diploid sectors was 11.0 $\mu \times 4.5\ \mu$. The diploid conidia were 1.7× more resistant to killing by ultraviolet light than were the haploid T9 conidia. Both the diploid colonies and their conidia were prototrophic. Diploids were very unstable, especially at 21°, and on supplemented minimal medium they readily formed sectors which had the haploid growth morphology and produced small conidia. Such sectors were usually auxotrophic for the requirements of the original heterokaryon components. As is shown in Table 4, these sectors often carried the recessive auxotrophic markers in new combinations. From all four of the diploids listed in this table at least one such recombinant type carried recessive markers from both heterokaryon mates.

DISCUSSION

The high frequency of auxotrophs among survivors of irradiated T9 conidia strongly suggests that this wild isolate is haploid. Other workers induced auxotrophs in a different strain of *Verticillium dahliae* (FORDYCE and GREEN 1964) and in several isolates of the closely related species *V. albo-atrum* (BUXTON and HASTIE 1962; HEALE 1966). Most wild-type isolates of *Verticillium* are haploid, although INGRAM (1968) reported a naturally occurring diploid.

The auxotrophs recovered after irradiation were stable in vegetative culture and were recovered from a parasexual cycle. Presumably they are the result of

single gene mutations in the nucleus. Definitive proof of this, however, is still lacking.

Complementary auxotrophs, when paired on minimal medium, produced colonies that resembled wild-type T9, except for slower growth. Once established, the heterokaryons on minimal medium were very stable, and apparently they could be perpetuated indefinitely. Growth rates varied from heterokaryon to heterokaryon and appeared to be related directly to the ratio of the two component nuclei. The more disparate the ratio, the slower was the growth rate. This growth was not the result of crossfeeding; hyphal contact was essential for growth. Because this same growth gave rise to heterozygous diploids, at least a transient heterokaryotic association was essential. Functionally, therefore, the prototrophic growth from pairings of complementary auxotrophs was heterokaryotic.

GIBSON and GRIFFIN (1958) suggested that heterokaryosis should also include mosaic colonies. *Nectria stenospora*, their test organism, had uninucleate hyphal cells with no evidence of nuclear migration. They contended that heterokaryosis could be restricted to a few anastomosed cells. Their data, however, were insufficient to establish this point.

Heterokaryosis in *V. dahliae* fits the ideas of GIBSON and GRIFFIN (1958). Most cells of the heterokaryon at 21° on minimal medium were uninucleate, including the tip cells. This finding agrees with those of TOLMSOFF (1973). The only binucleate cells that were consistently found were the H-shaped cells resulting from an anastomosis between two neighboring hyphae. The anastomosed cells appeared in the area where prototrophic mycelial sections were first isolated from samplings along the colony's radii (800–1000 μ back from the colony's edge). If such an anastomosis occurred between two hyphae that carried the two different nuclei of the heterokaryon, such cells would be heterokaryotic. We found no evidence for nuclear migration from one cell to another along a hypha or for the proliferation of the binucleate anastomosed cells. Heterokaryosis is apparently confined to the original anastomosed cell. This explains why the region of the hyphae between the anastomosed cells and the edge of the colony remains homokaryotic and therefore auxotrophic. Either one or both auxotrophic components of the heterokaryon can be isolated from this homokaryotic region. Where both components were recovered, however, they were segregated into separate hyphae.

Continued radial growth of the heterokaryotic colony required continued anastomosis. At 30°, hyphal anastomosis was greatly reduced and the heterokaryon stopped growing. Apparently at this high temperature the number of anastomoses was too meager to support the homokaryotic areas of the colony. Wild-type T9 grew at 30° because its cells were prototrophic.

Conidia from heterokaryotic colonies of *V. dahliae* were uninucleate. Any prototrophic conidium should be a rare heterokaryotic spore, a revertant to wild type, or a heterozygous diploid. Although up to 5×10^8 conidia from some heterokaryons grown at 21° were tested, no prototrophs were recovered. In the closely related species *V. alboatrum*, HASTIE (1964) found about one prototroph among 10^5–10^7 spores examined. These prototrophic spores were diploid.

When heterokaryons are incubated at 30° for over 8 days, sectors appear which are apparently diploid. This putative diploid may arise directly from the H-shaped anastomosed cells. The uninucleate anastomosed cells that were sometimes seen could have arisen from nuclear fusion within binucleate cells. Nuclear fusion was observed in an anastomosed cell. The morphology of the diploid is very distinct from that of the haploid. Conidia of the diploid were uninucleate, prototrophic, and twice as long as haploid spores. TOLMSOFF (1973) claims to have induced homozygous diploids in prototrophic isolates of *V. dahliae*. The conidia of these diploids were also uninucleate and twice as long as the conidia of their progenitors.

Diploid cultures were very unstable and formed sectors which carried the auxotrophic mutations present in the components of the heterokaryon from which the diploid arose. These sectors were probably haploid because they had the typical haploid morphology and produced small spores. Often the mutations were in new combinations. *V. dahliae*, therefore, has a parasexual cycle.

In contrast to heterokaryons of Neurospora, the heterokaryon of *V. dahliae* has the following features: (1) most hyphal cells are uninucleate except for a small, but important, number of binucleate anastomosed cells formed between hyphae; (2) there is no migration of nuclei from cell to cell; and (3) the tip cells of the colony edge remain homokaryotic. Heterokaryosis is apparently confined to the anastomosed cells, in which complementation between the auxotrophic components of the heterokaryon takes place. As a result, the anastomosed cells supply the necessary growth factors to the homokaryotic areas, which can be extensive. On minimal medium the heterokaryotic colony is a stable, integrated mosaic of homokaryotic and heterokaryotic regions.

Heterokaryosis as described here is probably not confined to *Verticillium dahliae*. Any fungus with uninucleate hyphal cells and frequent hyphal anastomosis is a likely candidate. Possible examples are *Verticillium albo-atrum* (HASTIE 1962), Fusarium species (BUXTON 1959), *Venturia inaequalis* (BOONE 1971), *Colletotrichum lagenarium* (DUTTA and GARBER 1960) and the common AB heterokaryon of *Schizophyllum commune* (MIDDLETON 1964).

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